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Phytoremediation: Green technology for the removal of mixed contaminants of a water supply reservoir

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Abstract

The Iraí Reservoir, a water supply in Brazil, is constantly impacted by anthropogenic activities such as waste inputs from agriculture, hospitals, and urbanization, resulting toxic cyanobacterial blooms causing economic, social, and environmental problems. The present study assessed the concentration of some common contaminants of the Iraí Reservoir, namely paracetamol, diclofenac, and microcystin-LR and tested whether a laboratory scale Green Liver System® would serve as a suitable technology to remove these contaminants. Further, the study investigated whether the pollutants caused adverse effects to the macrophytes using catalase as a biomarker for oxidative stress and investigated whether biotransformation (glutathione S-transferase) was a main route for detoxification. *Egeria densa*, *Ceratophyllum demersum*, and *Myriophyllum aquaticum* were exposed to a mixture of the three contaminants for 14 days in a concentration range similar to those detected in the reservoir. The plants removed 93 % of diclofenac and 100 % of MC-LR after 14 days. Paracetamol could not be detected. Catalase and glutathione S-transferase enzyme activities remained unaltered after the 14-day exposure, indicating that the mixture did not cause oxidative stress. The study showed that the aquatic macrophytes used are suitable tools to apply in a Green Liver System® for the remediation of mixed pollutants.

Keywords: Microcystin-LR, diclofenac, paracetamol, Green Liver System®, phytoremediation, aquatic macrophytes.

1. Introduction

Aquatic ecosystems are continuously affected by anthropogenic activities such as nitrogen and phosphorous inputs, mainly from agriculture, as well as other toxic compounds. These compounds can change the ecosystem dynamics, cause eutrophication, possibly resulting in cyanobacterial blooms which release toxins, all affecting the aquatic biota (Schulz et al. 2015; Scholz et al. 2017). Moreover, water contamination can result in human exposure via food and drink, thereby posing a threat to human health (Gibble et al. 2016). Water supply reservoirs are also affected by contamination resulting in high costs to the water treatment facilities to ensure safe drinking water (Calado et al. 2017).

The most commonly occurring cyanobacteria *Microcystis aeruginosa* is known to produce hepatotoxic microcystins (MCs), especially microcystin-LR (MC-LR) (Gupta et al. 2003; Omidi et al. 2018). Several studies have reported on the toxic effects of the MC-LR including liver failure in humans (Yuan et al. 2006), oxidative stress in aquatic organisms (Amado and Monserrat 2010), and molecular damage in mammalians (Zegura et al. 2011). In 1998, the World Health Organization established the limit for MC-LR (1000 ng/L) in drinking water (WHO 1998) and in Brazil the monitoring of cyanobacteria and cyanotoxins for water control was incorporated in 2000 (Brazil 2011). MCs are common in Brazilian reservoirs and studies have reported concentrations ranging from 0.5 to 4.5 μg/L (Fernandes et al. 2005; Ferrão-Filho et al. 2014; Hauser-Davis et al. 2015).

Due to wastage and only partial adsorption and metabolism of pharmaceuticals in humans, high concentrations of many pharmaceuticals have been detected in aquatic environments. Some pharmaceuticals cannot be completely removed by the current conventional water treatment processes and the population ingests these compounds via drinking water on a daily basis (Lonappan et al. 2016). Pharmaceuticals such as diclofenac, paracetamol, ibuprofen, and penicillin have been found in aquatic environments such as ground, and drinking water (Ebele et al. 2017; Yang et al. 2017).

Studies have reported that paracetamol can cause toxic effects in low concentrations (Nunes et al. 2014) and the effects in aquatic organisms have been reported (Guiloski et al. (2017b). Similarly, diclofenac is commonly found in aquatic ecosystems with numerous authors reporting accumulation in aquatic organisms causing damage (Cunha et al. 2017; Näslund et al. 2017, Liu et al. 2017; Gröner et al. 2017; Guiloski et al. 2017a). For this reason, the European Commission established the maximum allowed limit of 100 ng/L in drinking water and has declared diclofenac as a hazardous substance (European Commission 2012).

The Iraí Reservoir is located in the South of Brazil and is used as a potable water supply. There are many anthropogenic activities occurring around the reservoir such as agriculture, industries, hospitals, and settlements causing contamination and eutrophication leading to frequent cyanobacterial blooms. For this reason, several pharmaceutical, agricultural, and other chemical contaminants, including cyanobacterial toxins have been found in this water body (Bittencourt-Oliveira 2003; Kramer et al. 2015) like many others in the region. Due to the occurrence of these compounds in many reservoirs in developing countries, there a is a need for a low cost, sustainable, easy to manage, eco-friendly remediation technique ensuring safe drinking water.

Phytoremediation is a green technology used as a tool to improve and complement the water treatment processes. The Green Liver System® was recently reported as a suitable, sustainable, and environmentally friendly approach to remediate contaminated water bodies (Pflugmacher et al. 2015) showing success in the remediation of pharmaceuticals (Vilvert et al. 2017) and cyanobacterial toxins (Pflugmacher et al. 2016). It is a methodology that purifies water in a short time frame, using the uptake and biotransformation capacity of aquatic macrophytes. “Green Liver” in the name refers to the fact that the plants work as an animal liver in the biotransformation and detoxification of compounds (as detailed in Pflugmacher et al. 2015). However, to achieve efficient water purification it is necessary to replace the plants to avoid release of the metabolites (Pflugmacher et al. 2015).

In addition to evaluating the uptake of contaminants by plants, the effects on the plants also need to be assessed as mortality of the plants could lead to release of the contaminants. Several studies have investigated the activities of antioxidative enzymes, e.g. catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx), and biotransformation, as the activity of glutathione *S*-transferase (GST), as physiological biomarkers for adverse effects (Pflugmacher et al. 2007; Flores-Rojas et al. 2015; Spengler et al. 2017). These enzymes prevent cellular damage by degrading reactive oxygen species (ROS) such as $O_2\bullet$, H_2O_2 and $OH\bullet$, which are localized in chloroplast, mitochondria, and peroxisomes (Gill and Tuteja 2010). ROS can be produced under normal cellular metabolism or produced from xenobiotic exposure (Fernández-Fuego et al. 2017). GST, a biotransformation enzyme, conjugates electrophilic compound with glutathione (GSH), playing a role in the defense against oxidative damage (Van der Oost et al. 2003). Several studies have used this biomarker to evaluate the environmental stress in invertebrates, vertebrates, and plants (Pradhan et al. 2016; Lajayer et al. 2017). The use of these biomarkers is an advantageous tool in ecotoxicological studies and phytoremediation programs.

Based on the need for an eco-friendly, cost efficient remediation technology for developing countries i.e. to address the water quality issues of Brazil and based on the previous success reported with the Green Liver System® (Pflugmacher et al. 2015), the aims of the present study were therefore to (1) assess the concentration of the three drinking water contaminants of emerging concern in the Iraí Reservoir, Brazil, i.e. paracetamol (690 ng/L), diclofenac (12500 ng/L), and MC-LR (2030 ng/L), and to (2) evaluate the efficiency of the Green Liver System® to remove these three contaminants in the concentrations that were found in the reservoir, using *Egeria densa*, *Ceratophyllum demersum* and *Myriophyllum aquaticum* and to (3) assess the macrophytes' physiological responses to the exposure by monitoring the enzyme activities of CAT and GST.

2. Material and methods

2.1 Sampling from the Iraí Reservoir

Water samples were collected from the Iraí Reservoir on March 2017 using dark bottles with a total volume of 1 L according to Pierre Gy's theory of sampling principles (Pitard 1993). The water samples were frozen, concentrated by lyophilization (-48.3 °C, 0.1163 mbar), and resuspended in 70 % methanol before quantification via liquid chromatography tandem mass spectroscopy (LC-MS/MS). The yield of the compounds after lyophilization was evaluated before sample treatment. Three emerging contaminants, namely paracetamol, diclofenac, and MC-LR, were analyzed.

In short, acetaminophen (paracetamol) was quantified according to Esterhuizen-Londt et al. (2016), diclofenac was quantified according to Esterhuizen-Londt et al. (2017), and MC-LR was quantified according to Balsano et al. (2015).

2.2 Recovery after lyophilization procedure

Due to the low concentrations of the contaminants, an experiment to evaluate the compounds lost in the lyophilization procedure was carried out. It was tested using a control in Provasoli medium (Nimptsch et al. 2008) and a known concentration of the compounds (paracetamol: 690 ng/L, diclofenac; 12500 ng/L and MC-LR: 2030 ng/L). After total homogenization, water samples were frozen in liquid nitrogen, lyophilized, and re-diluted methanol (MS grade) followed by analyzed on LC-MS/MS. For the exposure experiments, it was decided to use a concentration 10 times higher than that quantified in Iraí Reservoir because of the percentage loss due to lyophilization and according to concentrations in Iraí Reservoir described in other studies.

2.3 Plant Material and chemicals

E. densa, *C. demersum*, and *M. aquaticum* were purchased from ExtraPlant (Extragroup GmbH, Münster, Germany). Aquatic macrophytes were maintained in tanks (100 L) over 7 days

148 for acclimation under controlled conditions, i.e. in pH 8 Provasoli media (Nimptsch et al. 2008),
149 at 20 ± 1 °C, and a photoperiod of 14 h light/10 h dark.

150 MC-LR was purchased from Alexxis GmbH (Grünberg, Germany). All other chemicals
151 were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany) unless stated
152 otherwise.

153 ***2.4 Exposure experiments***

154 Exposure experiments were carried out in a model Green Liver System®. The system
155 was built using glass with a total volume of 50 L. This system was divided into 6 compartments,
156 which are constructed to allow the continuous flow of water through the system via pumping
157 (as depicted in Nimptsch et al. 2008). *E. densa* was added to the first and second compartments
158 (n=4), *C. demersum* was added to the third and fourth compartments (n=4) and *M. aquaticum*
159 was added to the fifth and sixth compartments (n=4). Each plant species, which was fully
160 grown, had a mass of circa 150 g. Plants were exposed to 690 ng/L paracetamol, 12500 ng/L
161 diclofenac and 2030 ng/L MC-LR. The negative control consisted of the same conditions
162 without the addition of the compounds. The positive control consisted of running the system
163 with the contaminant mixture without plants. The experiment was performed during 14 days
164 and media samples were collected on day 0, 1, 3, 7 and 14. On day 14, all the plants samples
165 were collected. Five replicates were carried out for water and plant samples. During the
166 exposure, the Green Liver System® was kept under the same conditions as during
167 acclimatization.

168 The water samples collected were frozen in liquid nitrogen and lyophilized as stated
169 before. Afterwards, the samples were resuspended in 1 mL of methanol (MS-grade). The
170 samples were stored at –20 °C until LC-MS/MS analysis.

171 ***2.5 Extraction procedure and quantitative analysis***

172 Plants samples were collected on the end of the experiment in order to analyze the uptake
173 of the plants. Samples were frozen in liquid nitrogen and ground to a fine powder. Samples (0.1

174 g) were then added to MS-H_2O for paracetamol and 70 % methanol for diclofenac and MC-LR
175 extraction and left shaking for 30 min before centrifugation at 3400 × g for 10 min. The
176 supernatant was collected and the pellet were washed in an equal volume of MS-H_2O, followed
177 by vortexing and centrifugation at 3400 × g for 10 min. The supernatants were pooled and
178 filtered using 0.45 μm syringe cellulose acetate filters. The samples were stored at –20 °C until
179 the LC-MS/MS analysis. Both the media and the extracted samples were analyzed on LC-
180 MS/MS as stated in section 2.1.

181 ***2.6 Enzyme activities***

182 The activities of CAT (EC 1.11.1.6) and GST (EC 2.5.1.18) were analyzed in order to
183 assess the oxidative stress status and biotransformation in the plant tissues after exposure.

184 The enzymes were extracted as detailed by Pflugmacher (2004). In short, the plant
185 samples were ground in liquid nitrogen and 1.5 g of the powder was suspended in 3 mL of 0.1
186 mol/L sodium phosphate (NAP) buffer (pH 6.5) containing 1 mmol/L EDTA, 20 % (v/v)
187 glycerol, and 1.4 mmol/L dithioerythriol. The samples were stirred for 20 min on ice before
188 centrifugation at 5400 × g for 10 min at 4 °C. After a second centrifugation step (86900 × g, 60
189 min), the microsomal pellet was resuspended in 0.5 mL of 20 mmol/L NAP buffer containing
190 20 % glycerol. The 35 to 80 % saturation fraction was collected by NH_4SO_4 precipitation,
191 stirring for 20 min, followed by centrifugation at 48900 × g for 30 min. The supernatant was
192 discarded and the pellet was dissolved in 1 mL of 20 mmol/L NAP (pH 7.0). The samples
193 desalted on NAP 10 column (GE Healthcare Life, Freiburg Germany). Protein determination
194 was spectrophotometrically performed according to Bradford (1976), using Bradford's reagent.

195 CAT activity was measured according to Baudhuin et al. (1964) measuring the
196 breakdown of H_2O_2 (200 mmol/L) as substrate to H_2O and O_2 at 240 nm. GST activity was
197 evaluated according to Habig et al. (1974), measuring the conjugation of CDNB (1-chloro-2,4-
198 dinitrobenzen) and GSH (60 mmol/L) at 340 nm.

199 ***2.7 Statistical analysis***

The statistical analysis was performed using the R software 3.2.2 in order to compare the enzyme activities between the control and treatment plants. Levene's homogeneity test and Shapiro-Wilk normality preceded the data analysis. T-test was used to analyze the statistical differences between control and treatment plants. The significance level was $p < 0.05$.

205 **3. Results**

206 Paracetamol, diclofenac, and MC-LR were quantified in the reservoir water samples at
207 the concentrations of 69 ng/L, 1250 ng/L, and 203 ng/L respectively.

208 Lyophilization was used in the present study as a means to analyze the low
209 concentrations of the compounds present in Iraí Reservoir, which were below the lower limits
210 of detection of the LC-MS/MS methods. However, our results showed low recovery of the
211 compounds. The percentages of the recovery were: paracetamol <10 %, diclofenac 44.8 %, and
212 MC-LR 9.0 %. Therefore, the use of a 10-fold higher exposure concentration for the laboratory
213 experiments was selected.

214 Neither the water nor the plants of the negative control samples contained the tested
215 compounds. During the two-week exposure period, the aquatic macrophytes did not showed
216 any visible morphological alterations for neither the control nor the exposure sets.

217 ***3.1 Paracetamol***

218 Due to the quantification limit of the method, it was not possible to quantify paracetamol
219 in water samples. However, paracetamol in *M. aquaticum* was well measureable after 14 days
220 (137.6 ± 5.1 ng/g FW). The total amount taken up by the plants was 41.0 % of the exposure
221 concentration of 690 ng/L.

222 ***3.2 Diclofenac***

223 For the treatment samples, there was no significant decrease of the diclofenac
224 concentration in water samples after 7 days (Fig. 1). However, the diclofenac concentration in
225 the water samples decreased by 93.0 % after 14 days. When taking into account this result
226 compared to the control without plants, for which the degradation was 43.0 % in 14 days, this
227 is a significant reduction ($p < 0.05$). Diclofenac was measurable in *E. densa* (132.6 ± 30.1 ng/g
228 FW; 3.4 %) and *C. demersum* (160 ± 15.9 ng/g FW, 5.5 %). Of the total amount of diclofenac
229 (12500 ng/L), 8.9 % (1112.5 ng/L) was taken up by the macrophyte (Fig. 1).

230 Figure 1 here.

3.3 Microcystin-LR

The MC-LR concentrations in water samples from the treatment set decreased by 69 % within 24 h and 100 % after 3 days (Fig. 2). In the parallel experiment without plants, the degradation was 55 % after 7 days and 61 % after 14 days. The concentrations in plants were below of the quantification limit (Fig. 2).

Figure 2 here.

3.4 Enzyme activities

CAT and GST activities were not significantly different between control and treatments in *E. densa* (t=0.2135, p=0.8414; M=4, p=1), *C. demersum* (t=-18883, p=0.1321; t=0.5125, p=0.6363), or *M. aquaticum* (t=1.5797, p=0.1888; t=-0.2736, p=0.7979) (Fig. 3).

Figure 3 here.

4. Discussion

The results of the chemical analysis showed that Iraí Reservoir is contaminated with a mixture of compounds including diclofenac, paracetamol, and MC-LR. The concentrations of these compounds can be associated with the anthropogenic activities around this water body. Although this reservoir is used as a water supply, there are several anthropogenic activities that contribute to input of pharmaceuticals and cyanotoxins in the water. The hospital and settlements contribute to inputs of the pharmaceuticals in water (Santos et al. 2010), and the agriculture activities contribute to the increase of nutrients and organic matter result in cyanobacterial blooms (Scholz et al. 2017).

The measured MC-LR concentration (203 ng/L) was below of the legislation limit for drinking water (1000 ng/L) (Brazil 2011); however, the diclofenac concentration (1250 ng/L) was ten times more than that allowed by the legislation for drinking water (100 ng/L) (European Commission 2012). Paracetamol is a pharmaceutical that is not incorporated in Brazilian or European legislation. Since pharmaceuticals are present in water bodies at high concentrations, it is important to regulate these emerging contaminants. These contaminants may cause

problems to the aquatic organisms and human health, and are particularly worrisome since they cannot be totally removed by conventional water treatment (Lonappan et al. 2016; Guiloski et al. 2017b).

Although minute concentrations of these compounds can pose a risk to the environment, these low amounts are difficult to quantify. For developing countries restricted by their financial dispositions, it is not possible to use state of the art, highly efficient methods as they are often very expensive to implement. The lyophilization, selected as it is an inexpensive method, proved to be inefficient to concentrate environmental concentrations to those analyzable on LC-MS/MS. Furthermore, it means that, the concentration found in Iraí Reservoir can be underestimated. Fonte et al. (2006) assessed the lyophilization procedure and reported that the processing conditions can result in freezing and desiccation stress causing damage and instability to the substances. In addition, any change in the process can transform an efficient into inefficient process. In future studies, other methodologies to improve the recovery to the quantification of compounds should be tested.

In the Green Liver System® experiment, paracetamol could not be quantified in water samples, however, 41 % of the total amount of paracetamol could be quantified intracellularly in *M. aquaticum* suggesting uptake. The other 59 % could have been biotransformed by the plants, and/or by natural/bacterial degradation, surface bound or have not been up taken or degraded. Paracetamol transformation can form metabolites such as N-acetyl-benzoquinomine, glucuronide, sulfate, and mercapturate; and the biotransformation by plants can be via glucoronisation and generation of conjugates with glutathione (Huber et al. 2009). Another study that tested plants to remove paracetamol, but using wetlands, showed that paracetamol was removed, however, it was attributed more to the degradation associated to the biofilm in roots (Ranieri et al. 2011).

Diclofenac was reduced by 93 % in water samples and only 43 % for natural and/or bacterial degradation after 14 days, suggesting that the plants are taking up this compound. The

283 results obtained are comparable to those achieved by Matamoros et al. (2012) within the same
284 time frame using only *C. demersum*. However, diclofenac concentrations in *E. densa* and *C.*
285 *demersum* tissues were only 8.9 % of the total amount and this result can be possibly attributed
286 to the biotransformation of diclofenac intracellularly.

287 Diclofenac can be transformed into several products such as diclofenac-lactam, 4'-
288 hydroxy-diclofenac, 5'-hydroxy-diclofenac, and diclofenac-benzonic acid. Studies have
289 suggested than transformation and biotransformation can occur via monooxygenation,
290 oxidation, decarboxylation, conjugation, and hydroxylation (Jewell et al. 2016; Bouju et al.
291 2016) and that the process can occur very rapidly; for example, Huber et al. (2012) reported
292 biotransformation after 3 h. Diclofenac and its metabolites were quantified in the plant tissues;
293 and after 7 days 66 % of diclofenac concentrations decreased in the plants. They also suggest
294 the biotransformation of diclofenac is via hydroxylation to 4'-hidroxy-diclofenac and
295 conjugation to glucopyranoside.

296 For the treatment samples, after 3 days in the presence of the plants, no MC-LR could
297 be detected, and in the control experiment the concentration remained stable for the first 3 days.
298 This result suggested that the plants took up MC-LR and can be used as tools in the pretreatment
299 of water from reservoirs that are constantly contaminated with MCs. The MC-LR
300 concentrations in plants were below of the quantification limit. However, other studies that used
301 5 to 10 times higher concentrations showed that MC-LR was up taken by the aquatic plants and
302 *C. demersum* was a successful plant to remove this toxin (Pflugmacher et al. 2015; Contardo-
303 Jara et al. 2015). In a study by Romero-Oliva et al. (2015) it was shown that *E. densa* had a
304 higher MCs bioaccumulation capability compared by *C. demersum*. The low concentrations
305 used in the present study may have resulted in low measurement in the plant tissue. In addition,
306 studies described that when MC-LR enter the cell, it binds to GSH and phosphatase proteins
307 (Bittencourt-Oliveira et al. 2013; Liu and Sun 2015) and the method used only quantifies free

MC-LR. However, it should be considered that after 14 days the MC-LR could have been biotransformed in the plants.

E. densa, *C. demersum*, and *M. aquaticum* took up the composts differently. Diclofenac was measured in *E. densa* and *C. demersum*, yet paracetamol could be only measured intracellulary in *M. aquaticum*. This result showed the importance of working with different plant species in phytoremediation programs, mainly in aquatic environments that are contaminated by a mixture of compounds. In this context, different plants species respond in different ways to the contaminants and the choice of the species used in phytoremediation is a very important step.

Studies that evaluate the stress in plants, report the sensitivity of these organisms to contaminants and it can determine which species can be used as bioindicators or for phytoremediation. GST and cytochrome P450 enzymes can participate in the biotransformation of paracetamol, diclofenac, and MC-LR. However, in the present study, the GST activities of the exposed plants were not elevated when compared to the control. It can be due to analysis only being conducted after 14 days and this enzyme's activity could have returned to normal levels. Pflugmacher (2004) showed that in *C. demersum*, GST levels started to reduce after 48 h of exposure to 0.5 ng/L Mc-LR. Nunes et al. (2017), in a study with clams, reported that the GST enzyme was activated in the first 96 h of the paracetamol exposure. After 10 days the GST activity was similar among control and treatment groups. In addition, other biotransformation enzymes that were not checked in the present study can be metabolizing these compounds such as P450 monooxygenases and glycosyltransferases (Huber et al. 2012). Another study that evaluated biotransformation of endocrine disrupting chemicals in *C. demersum*, suggested high efficiency in the peroxidases metabolism for detoxification when compared with the GST metabolism (Reis et al. 2014).

In the present study, CAT activity also was not statistically different between control and treatment plants. This result suggests that the CAT activity was not elevated at all or

returned to normal after 14 days in the plant species at the concentrations used in this study. Studies using *Hydrilla verticillata* (Spengler et al. 2017) and *Fucus vesiculosus* (Pflugmacher et al. 2007) have reported the increase of the CAT activity after exposure to contaminants. In addition, Kummerová et al. (2016), evaluating paracetamol and diclofenac exposure in *Lemna minor* showed induction of stress oxidative, increase of GST activity, and low cell viability of the roots.

From the data obtained, *E. densa, C. demersum,* and *M. aquaticum* were efficient in the removal of the contaminants in the water and this system can be a successful tool to use in water supply reservoirs. Two experimental large-scale Green Liver System® were built in China and in Northeast of Brazil. Both experiments showed an excellent performance with an uptake higher than 80 % for cyanotoxins and an antibiotic (Pflugmacher et al. 2015). According to the current situation of Iraí Reservoir, the application of the Green Liver System® could be an alternative to solve the problem of contamination, reduce costs for the water treatment, and reduce the risk to human health.

5. Conclusion

Paracetamol, diclofenac, and MC-LR were quantified in the Iraí Reservoir and these compounds can pose a risk to environmental and human health. In the present study, the compounds were taken up by the plants tested and no oxidative stress incitement was evident after 14 days. Therefore, *E. densa*, *C. demersum*, and *M. aquaticum* are deemed as suitable tools to use in phytoremediation. In addition, the Green Liver System® is a sustainable method that could be applied to improve the drink water treatment.

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Declaration of interest:

Conflict of interest: none.

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Figure captions

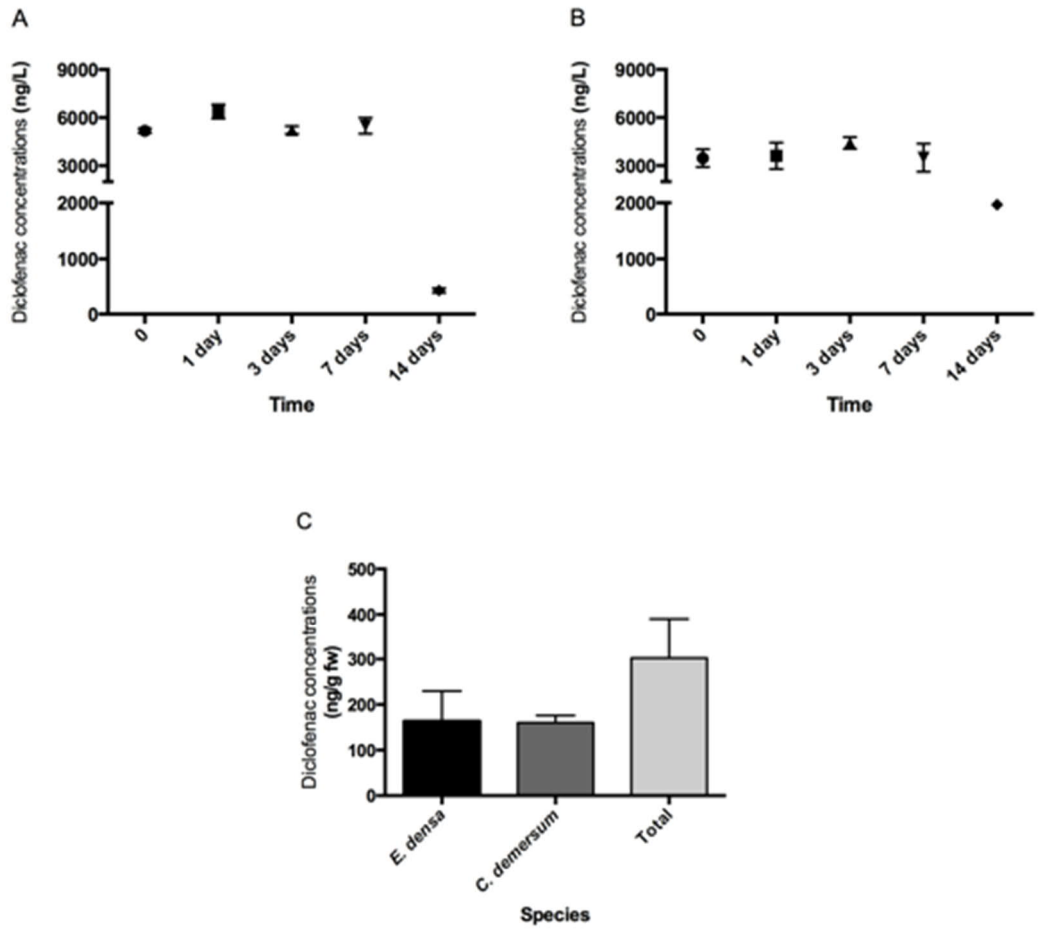
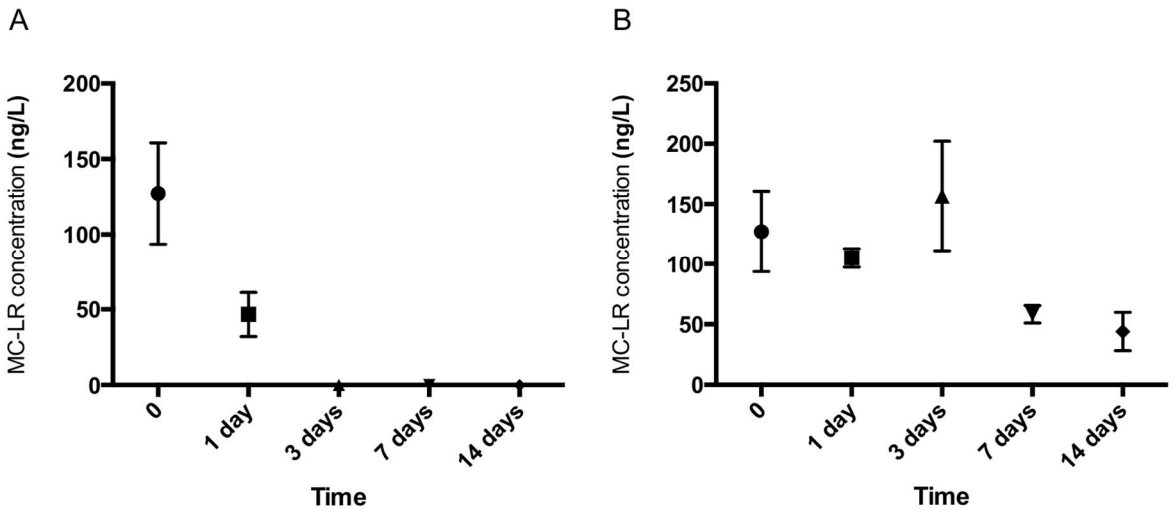


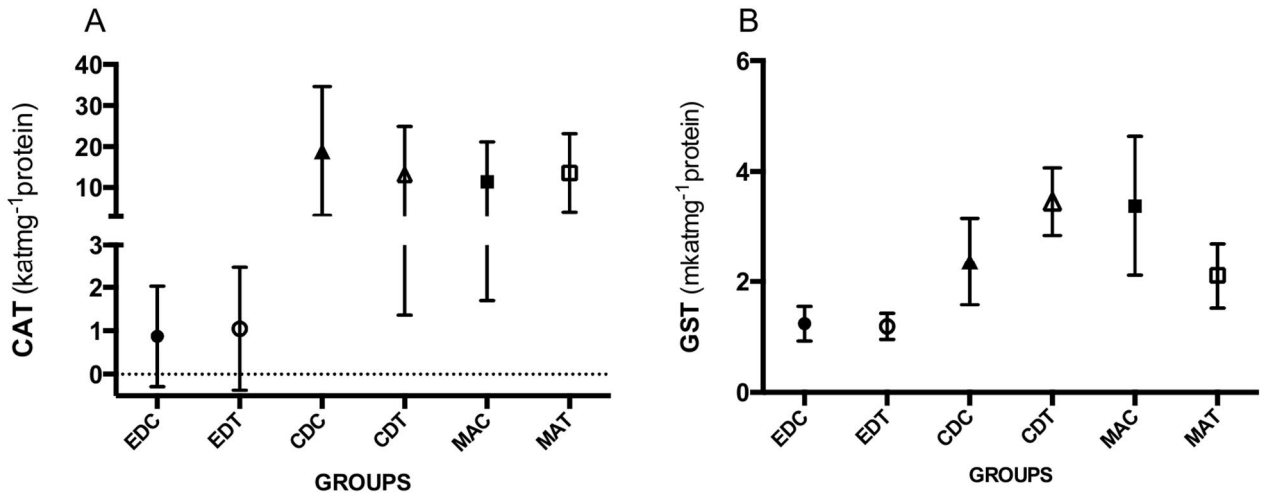
Figure 1. Diclofenac concentration: (**A**) Experiment Green Liver System®; (**B**) Experiment control without plants and (**C**) plant species from experiment Green Liver System®. Mean ± SD (n=5).



573

574 **Figure 2.** MC-LR concentrations: (**A**) Experiment Green Liver System®; (**B**) Experiment

575 control without plants. Mean ± SD (n=5).



576

577 **Figure 3.** CAT activity (**A**) and GST activity (**B**) in plant tissues with exposure. Data points

578 represent mean ± SD (n=3); p=<0.05. **EDC**: *E. densa* control; **EDT**: *E. densa* treatment;

579 **CDC**: *C. demersum* control; **CDT**: *C. demersum* treatment; **MAC**: *M. aquaticum* control;

580 **MAT**: *M. aquaticum* treatment.